Transport of Pyruvate nad Lactate into Human Erythrocytes

EVIDENCE FOR THE INVOLVEMENT OF THE CHLORIDE CARRIER AND A CHLORIDE-INDEPENDENT CARRIER

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The kinetics and activation energy of entry of pyruvate and lactate into the erythrocyte were studied at concentrations below 4 and 15 mM respectively. The K_m and $V_{max.}$ values for both substrates are reported, and it is shown that pyruvate inhibits competitively with respect to lactate and vice versa. In both cases the K_m for the carboxylate as a substrate was the same as its K_i as an inhibitor. α-Cyano-4-hydroxycinnamate and its analogues inhibited the uptake of both lactate and pyruvate competitively. Inhibition was also produced by treatment of cells with fluorodinitrobenzene but not with the thiol reagents or Pronase. At high concentrations of pyruvate or lactate (20 mM), uptake of the carboxylate was accompanied by an efflux of Cl^- ions. This efflux of Cl^- was inhibited by α-cyano-4-hydroxycinnamate and picrate and could be totally abolished by very low (<10 μM) concentrations of the inhibitor of Cl^- transport, 4,4'-di-isothiocyanostilbene-2,2'-disulphonic acid. This inhibitor titrated out the chloride efflux induced by pyruvate, bicarbonate, formate and fluoride, in each case total inhibition becoming apparent when approximately 1.2×10^6 molecules of inhibitor were present per erythrocyte, that is, about one inhibitor molecule per molecule of the Cl^- carrier. Even when Cl^- efflux was totally blocked pyruvate and lactate uptake occurred. Kinetic evidence is presented which suggests that the Cl^- carrier can transport pyruvate and lactate with a high K_m and high $V_{max.}$, but that an additional carrier with a low K_m and a low $V_{max.}$ also exists. This carrier catalyses the exchange of small carboxylate anions with intracellular lactate, is competitively inhibited by α-cyano-4-hydroxycinnamate and non-competitively inhibited by picrate. The Cl^- carrier shows a reverse pattern of inhibition. It is concluded that net efflux of lactic acid from the cell must occur on the Cl^- carrier and involve exchange with HCO_3^- followed by loss of CO_2. The low K_m carrier might be used in pyruvate/lactate or acetoacetate/β-hydroxybutyrate exchanges involved in transferring reducing power across the cell membrane. The possibility that the Cl^- carrier exists in cells other than the erythrocyte is discussed. It is concluded that its presence in other cell membranes together with a low intracellular Cl^- concentration would explain why the pH in the cytoplasm is lower than that of the blood, and why permeable carboxylate anions do not accumulate within the cell when added from outside.

Pyruvate and lactate are both able to cross the erythrocyte membrane, and may be accumulated within the cell if a Cl^- gradient is present (Watts & Randle, 1967; Halestrap & Denton, 1974, 1975). Evidence that this transport across the membrane involves a specific carrier was obtained by using the inhibitor α-cyano-4-hydroxycinnamate and its analogues (Halestrap & Denton, 1974, 1975). These compounds are also inhibitors of mitochondrial pyruvate transport, where they exhibit much more potent inhibitory activity than is seen with erythrocytes (Halestrap, 1975; Halestrap & Denton, 1975).

In the present paper I examine the mechanism of transport of lactate and pyruvate into erythrocytes. The kinetic properties of the transport process for both substrates are described, and the interactions of

various inhibitors reported. It is shown that lactate and pyruvate enter the erythrocyte as anions, either by using the well-established and non-specific anion transporter involved in transport of chloride, sulphate, phosphate and bicarbonate (see Deuticke, 1972; Passow & Wood, 1974; Sachs *et al.*, 1975; Ho & Guidotti, 1975), or on a more specific carboxylate transporter that has a much lower K_m and $V_{max.}$ than the general anion transporter. The properties of both these carriers have been studied, and it is concluded that net transport of lactic acid out of the cell must involve the general anion transporter, a bicarbonate/CO_2 cycle being used to transport the proton (see Scheme 1). However, pyruvate/lactate or acetoacetate/β-hydroxybutyrate exchange can occur on the more specific carrier, and it is suggested that this

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might be of importance in the transport of reducing equivalents between the plasma and the cytoplasm. The implications of anion transport across the cell membrane on pH and metabolite distribution are also discussed.

Experimental

Materials

Erythrocytes. These were obtained from fresh or recently outdated human blood, and were normally washed twice in 4vol. of 0.15M-NaCl before use. Unless otherwise stated, cells were washed a further three times in 4vol. of citrate buffer (84mM-sodium citrate/10mM-Tris, adjusted to pH7.4 with NaH_2PO_4) at 4°C, before being made up as a 25% suspension in the same buffer.

Chemicals. N-Ethylmaleimide and 4-acetamido-4'-isothiocyanostilbene-2,2'-disulphonic acid were obtained from BDH Chemicals Ltd., Poole, Dorset, U.K. Fluorodinitrobenzene and acetazolamide were from Sigma (London) Chemical Co., London S.W.6, U.K. All radiochemicals were from The Radiochemical Centre, Amersham, Bucks., U.K. 4,4'-Diaminostilbene-2,2'-disulphonic acid and thiophosgene were from Aldrich Chemical Co., Wembley, Middx., U.K., and Pronase was from Calbiochem Ltd., London W1H 1AS, U.K. 4,4'-Di-isothiocyanostilbene-2,2'-disulphonic acid was prepared from 4,4'-diaminostilbene-2,2'-disulphonic acid and excess of thiophosgene as described by Maddy (1964). The product was freed of reaction products by freeze-drying, and tested for purity by electrophoresis and i.r. spectroscopy. Paper electrophoresis was carried out at pH6.5 [10% (v/v) pyridine, 1% (v/v) acetic acid in water] and pH1.6 [1.5% (w/v) formic acid in water] for 45min at 3kV. Both 4,4'-di-isothiocyanostilbene-2,2'-disulphonic acid and 4-acetamido-4'-isothiocyanostilbene-2,2'-disulphonic acid showed distinct differences in mobility from 4,4'-diaminostilbene-2,2'-disulphonic acid at both pH6.5 and 1.6 and showed no appreciable impurities when viewed by u.v. fluorescence. I.r. spectra confirmed the complete conversion of amino groups into isothiocyano groups in 4,4'-di-isothiocyanostilbene-2,2'-disulphonic acid.

Sources of all other chemicals and biochemicals are as given by Halestrap (1975).

Methods

Measurement of uptake of pyruvate and lactate. Uptake of pyruvate and lactate into erythrocytes was measured radioactively as described previously (Halestrap & Denton, 1974). Uptake was stopped when required by the addition of 2.5mM-α-cyano-4-hydroxycinnamate before separation of the cells by

centrifugation. Unless otherwise stated incubations were performed at 10°C. Chloride exchange across the cell membrane was measured similarly but at 2.5°C and with $Na^{36}Cl$ (0.1μCi/ml). Exchange was terminated by addition of 4,4-di-isothiocyano-2,2'-stilbenedisulphonic acid (20μM) before centrifugation and analysis of the cells for ^3H and ^{36}Cl. [^3H]-Inulin (1μCi/ml, 50μg/ml) was used throughout to correct for the extracellular space, and $^{14}C/^3H$ and $^{36}Cl/^3H$ dual-label counting was performed on an Isocap 300 liquid-scintillation counter by using the necessary quench corrections. A methoxyethanol/toluene scintillant was used as described previously (Halestrap & Denton, 1974). The intracellular volume was determined by using ^3H_2O and [U-^{14}C]sucrose as described by Halestrap & Denton (1974).

Measurement of Cl^- and proton fluxes. Cl^- fluxes were studied with a Cl^- electrode (a silver electrode coated with AgCl) in a constant-temperature water-jacketed container connected to a reference calomel electrode by a salt bridge. The cells were added as a 25% suspension in citrate buffer and continuously stirred with a magnetic stirrer. Output of Cl^- ions was determined from the change in potential measured with a Radiometer model 26 pH-meter connected to a chart recorder (1mV full-scale deflexion) and calibrated with KCl. All additions made to the cell suspension were checked for interference with the Cl^- electrode (ions such as Br^-, I^- and SCN^- interfere). Proton fluxes associated with carboxylate entry into the cells were measured similarly, but cells were washed and suspended in 90mM-Na_2SO_4/10mM-sodium citrate/2mM-Tris, adjusted to pH7.0 with NaH_2PO_4, and the Cl^- electrode was replaced by a glass electrode. Calibration of proton efflux was performed by addition of standardized KOH.

Haematocrits were determined by measuring the supernatant volume after centrifuging at $15000g_{av}$ for 1min.

Results and Discussion

Studies on the uptake of pyruvate and lactate into erythrocytes

The kinetics of the transport of pyruvate and lactate into erythrocytes were studied using the 'inhibitor-stop' technique that has been used extensively in the study of mitochondrial metabolite transport (see Klingenberg, 1970) and sugar transport into erythrocytes (see Jung, 1975). α-Cyano-4-hydroxycinnamate was shown to be a suitable inhibitor to terminate the transport of both pyruvate and lactate into erythrocytes immediately and completely. Thus at 2.5mM-α-cyano-4-hydroxycinnamate and a substrate concentration of 0.2mM no detectable uptake of pyruvate or lactate by erythrocytes was observed in 5min at 10°C, whether the inhibitor was added before

or at the same time as the substrate. If uptake of pyruvate was permitted in the absence of inhibitor for 1 min and then 25 mM-α-cyano-4-hydroxycinnamate was added, the amount of pyruvate within the erythrocyte remained unchanged for a further 5 min (0.362 ± 0.014 and 0.381 ± 0.013 nmol/μl of intracellular space at 1 and 6 min respectively; values are means$\pm$S.E.M. for four observations). At very high substrate concentrations (>5 mM) inhibition by 2.5 mM-α-cyano-4-hydroxycinnamate was still substantial (>90%) but not complete, probably because inhibition is competitive as is shown below. However, provided centrifugation was performed rapidly after addition of the inhibitor, the uptake of additional substrate was not significant. By using these conditions, the time-course of pyruvate and lactate uptake into erythrocytes was investigated. At all substrate concentrations used (0.1–20 mM) uptake was linear for at least 1 min at 10°C. Thus for kinetic studies the amount of substrate taken up in the first minute was taken to be the initial rate of transport. All substrate concentrations used are expressed with respect to the extracellular volume and not the total incubation volume.

The uptake of pyruvate and lactate into erythrocytes at concentrations of less than 4 and 15 mM respectively followed conventional Michaelis–Menten kinetics. The results of eight pyruvate and three lactate experiments at 10°C, each involving at least 24 observations, gave $V_{max.}$ values for pyruvate and lactate of 2.01 ± 0.05 and 1.95 ± 0.09 nmol/min per μl of intracellular space respectively, and K_m values of 1.89 ± 0.06 and 9.14 ± 0.53 mM respectively. The nature of the inhibition caused by various compounds and the accompanying K_i values are reported in Table 1. It is noteworthy that the K_i for the competitive inhibition of pyruvate transport by lactate (11.92 ± 1.33 mM) and the K_i for the competitive inhibition of lactate transport by pyruvate (1.64 ± 0.17 mM) are very similar to their K_m values as substrates for transport. This implies that the K_m values represent the dissociation constant of the transporter–substrate complex and therefore that transport rather than binding is the rate-limiting process (see Dixon & Webb, 1958).

Inhibition of transport by α-cyano-4-hydroxycinnamate is also competitive, with a K_i of about 50–60 μM for transport of both lactate and pyruvate. This contrasts with mitochondrial pyruvate transport, where inhibition is non-competitive, and probably involves the formation of a reversible covalent bond with an active thiol group on the transporter (Halestrap, 1975; A. P. Halestrap, unpublished work). The potent activity of α-cyanocinnamate derivatives on mitochondrial pyruvate transport requires the presence of the α-cyano group adjacent to the double bond (Halestrap, 1975), and this appears to be necessary to make the compound reactive towards thiol groups (A. P. Halestrap, unpublished work). How-

ever, in the erythrocyte the α-cyano group can be replaced by a fluorine atom (as in α-fluorocinnamate) without appreciable loss of inhibitory activity, although replacement by a hydrogen atom (as in 4-hydroxycinnamate) considerably decreases inhibition (Table 2). Thus it appears that there are considerable differences in the mode of inhibition by α-cyano-4-hydroxycinnamate of the transport of pyruvate into erythrocytes from that into mitochondria. One is competitive and non-covalent, the other is non-competitive and involves covalent modification of a thiol group. However, in both cases a hydrophobic grouping such as a benzene or indole ring greatly enhances inhibitory activity (see Table 2, and Halestrap, 1975), presumably by binding to a nearby hydrophobic region of the protein or membrane lipid. In the case of the erythrocyte, the presence of a hydroxyl group on the benzene ring does not impair inhibition as it does with mitochondria. With both transport processes the most potent inhibitor found was α-cyano-β-(1-phenylindol-3-yl)-acrylate, which in the erythrocyte showed mixed inhibition with both $K_i(I)$ and $K_i(II)$ between 10 and 20 μM. However, this is still nearly three orders of magnitude less potent than it is towards mitochondrial pyruvate transport.

Further evidence that α-cyanocinnamate derivatives do not inhibit erythrocyte pyruvate transport by attacking an essential thiol group comes from the observation that thiol reagents such as iodoacetamide, iodoacetic acid and N-ethylmaleimide were all without appreciable effect (Table 2), in contrast with their inhibition of mitochondrial pyruvate transport (Papa & Paradies, 1974). However, an amino reagent, fluorodinitrobenzene, at 1 mM totally blocked pyruvate entry into the erythrocyte (Table 2). This would imply the involvement of amino groups in transport of pyruvate and lactate across the erythrocyte membrane, a requirement that is also present for the transport of other anions on the general anion transporter (see Sachs et al., 1975). Various other inhibitors of pyruvate transport are shown in Tables 1 and 2 that indicate that a large variety of carboxylates can inhibit pyruvate entry into erythrocytes. They include phenylpyruvate and 2-oxo-4-methylpentanoate, which are involved in the pathology of phenylketonuria and maple-syrup-urine disease respectively. It has been suggested that the inhibition of mitochondrial pyruvate transport by these compounds may be an important aspect of the pathology of the diseases (Halestrap et al., 1974). It is possible that inhibition at the level of cellular transport of pyruvate and lactate may also be important.

In Fig. 1 the temperature-dependence of the uptake of pyruvate into erythrocytes is shown. At about 10°C there is a definite change in slope of the Arrhenius plot, but on either side of this temperature the plot is linear, yielding an activation energy of

Table 1. *Kinetic parameters of the transport of pyruvate and lactate into erythrocytes*

Erythrocytes, suspended in citrate buffer at about 25% haematocrit and containing 1 μCi of [^{3}H]inulin/ml (50 μg/ml), were incubated at 10°C for 5 min before addition of ^{14}C-labelled substrate at the desired concentration and at a specific radio-activity of 0.1 μCi/μmol at pyruvate and lactate concentrations of less than 1 and 2.5 mM respectively, and 0.025 μCi/μmol at concentrations higher than these. Uptake of substrate was terminated after 1 min by addition of 2.5 mM-α-cyano-4-hydroxy-cinnamate. The cells were then centrifuged and analysed for ^{14}C-labelled substrate as described under 'Methods'. Where present, inhibitors were added with the substrate. Inhibitor constants were computed by least-squares regression and are expressed as the value±S.E.M. for at least 12 observations in the presence and absence of inhibitor. The type of inhibition shown was determined from which of the following equations (taken from Cleland, 1963) gave the most significant fit:

$$\text{Mixed inhibition, } v = \frac{V_{max.}}{1+\frac{K_m}{[S]}\left[1+\frac{[I]}{K_i(I)}\right]+\frac{[I]}{K_i(II)}} \qquad\qquad \text{Uncompetitive inhibition, } v = \frac{V_{max.}}{1+\frac{K_m}{[S]}+\frac{[I]}{K_i}}$$

$$\text{Competitive inhibition, } v = \frac{V_{max.}}{1+\frac{K_m}{[S]}\left[1+\frac{[I]}{K_i}\right]} \qquad\qquad \text{Non-competitive inhibition, } v = \frac{V_{max.}}{\left[1+\frac{K_m}{[S]}\right]\left[1+\frac{[I]}{K_i}\right]}$$

Those inhibitors which exhibited partially uncompetitive inhibition have not been given K_i values; this matter is discussed in the text.

			K_m value or inhibitor constants (mM)	
Substrate	Inhibitor	Type of inhibition	$K_i(I)$	$K_i(II)$
Pyruvate	None	K_m value	1.89 ±0.06	
	Lactate	Competitive	11.92 ±1.33	—
	α-Cyano-4-hydroxycinnamate	Competitive	0.049 ±0.004	—
	α-Cyanocinnamate	Competitive	0.088 ±0.006	—
	α-Cyano-β-(1-phenylindol-3-yl)acrylate	Mixed	0.0123±0.002	0.019±0.003
	Phenylpyruvate	Competitive	0.60 ±0.06	—
	2-Oxo-4-methylpentanoate	Competitive	1.34 ±0.11	—
	Formate, chloride, picrate, bicarbonate or 4,4'-Di-isothiocyanostilbene-2,2'-disulphonic acid	Partially uncompetitive	—	—
Lactate	None	K_m value	9.14 ±0.53	—
	Pyruvate	Competitive	1.64 ±0.17	—
	α-Cyano-4-hydroxycinnamate	Competitive	0.064 ±0.006	—

Table 2. *Inhibition of pyruvate entry into erythrocytes by various compounds*

Pyruvate uptake was measured radioactively as described in Table 1 and under 'Methods'. Uptake was terminated after 1 min by addition of α-cyano-4-hydroxycinnamate (2.5 mM). In Expt. I, pyruvate was present at 2.5 mM and inhibitors at 0.25 mM. The temperature was 15°C. In Expt. II inhibitors were preincubated with the erythrocytes for 15 min at 37°C before pyruvate uptake was measured at 10°C and 1.3 mM-pyruvate; control cells were preincubated similarly, but in the absence of inhibitor. Results are means of two observations agreeing within 5% (Expt. I) or means±S.E.M. of four observations (Expt. II).

| Expt.	Inhibitor	Pyruvate uptake (nmol/min per μl of intracellular space)	Inhibition (%)
I	None	2.27	0
	α-Cyanocinnamate	0.94	59
	α-Cyano-4-hydroxycinnamate	1.00	56
	α-Cyano-5-phenylpenta-2,4-dienoate	1.11	51
	α-Cyano-β-(1-phenylindol-3-yl)acrylate	0.29	87
	α-Cyano-4-methylpentanoate	1.94	15
	α-Fluorocinnamate	0.90	60
	4-Hydroxycinnamate	1.87	18
	α-Bromophenylacetate	1.60	30
	α-Thio-2-furanpyruvate	1.65	27
II	None	1.07±0.02	0
	Iodoacetate (5 mM)	0.95±0.02	11
	Dinitrofluorobenzene (1 mM)	0.12±0.01	90
	N-Ethylmaleimide (0.5 mM)	1.08±0.02	0
	Iodoacetamide (2.0 mM)	0.83±0.01	22
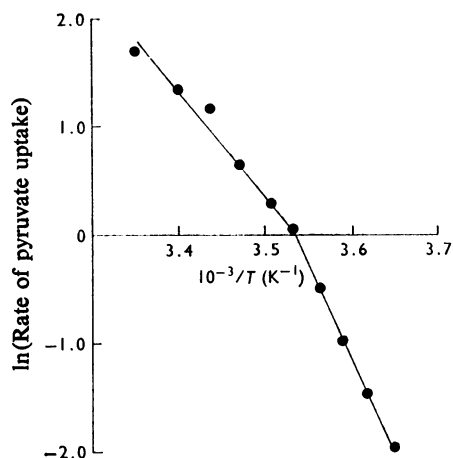
Fig. 1. *Temperature-dependence of pyruvate transport into erythrocytes*

Pyruvate uptake was measured radioactively as described in Table 1 and under 'Methods', and at the temperature shown. Uptake was terminated at 3 min (1–3.5°C), 1.5 min (5.5–7.5°C), 1 min (10–15°C) and 30 s (18–25.5°C) to ensure that initial uptake only was measured. [U-^{14}C]Pyruvate was present at 1.5 mM and 0.1 μCi/ml. Linear regression of the Arrhenius plot shown gave values for the activation energy of 148±4 kJ/mol (0–10°C) and 77±6 kJ/mol (10–25.5°C).

148±4 kJ/mol between 0 and 10°C and 77±6 kJ/mol between 10 and 25°C. Such a break in the activation energy at about 10°C was reported in some mitochondrial transport systems (Klingenberg, 1970; Halestrap, 1975) and may involve a membrane phase transition. The high value for the activation energy of pyruvate transport into erythrocytes between 0 and 10°C is also a feature of chloride transport under the same conditions, where the activation energy was calculated to be 139 kJ/mol (Dalmark & Wieth, 1970; Dalmark, 1972). Extrapolation from the $V_{max.}$ and activation energy gives a maximal rate of pyruvate transport into or out of the erythrocyte at 37°C of about 32 nmol/min per μl of intracellular space.

Cl$^-$ efflux associated with carboxylate entry into erythrocytes

The experimental conditions used to produce pyruvate accumulation by erythrocytes create a Cl$^-$ gradient, since the cells are suspended in a Cl$^-$-free medium containing the impermeant anion, citrate. It was therefore decided to establish the relationship between pyruvate uptake and Cl$^-$ efflux from the cell; this was done by the use of the Cl$^-$ electrode, which allowed a continuous record of Cl$^-$ efflux to be made.
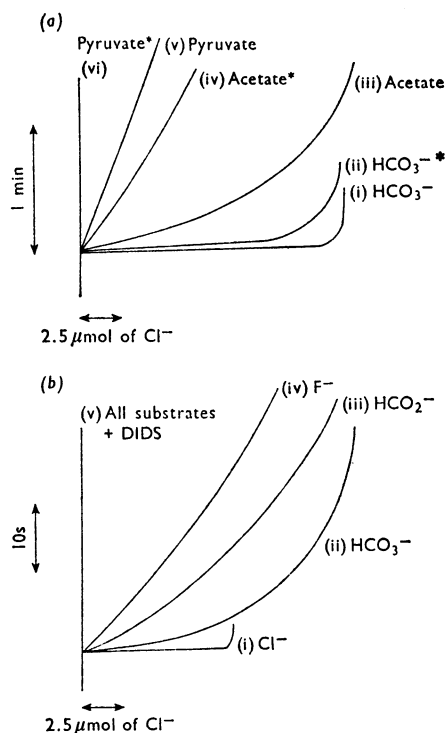
Fig. 2. *Cl$^-$ efflux associated with anion entry into the erythrocyte*

Erythrocytes in citrate medium (25% haematocrit) were equilibrated at 15°C (*a*) or 2.5°C (*b*) in a Cl$^-$-electrode chamber (see under 'Methods') and Cl$^-$ efflux was initiated by the addition of substrate. Calibration was performed by addition of a standard KCl solution. Additions were as follows: (*a*) 20 mM-NaHCO$_3^-$ (i, ii), 20 mM-sodium acetate (iii, iv) or 20 mM-sodium pyruvate (v, vi); an asterisk denotes that α-cyano-4-hydroxycinnamate (2 mM) was also present in Expts. (ii), (iv) and (vi); (*b*) (i) 10 μmol of KCl, (ii) 20 mM-NaHCO$_3$, (iii) 20 mM-sodium formate, (iv) 20 mM-NaF, (v) all substrates+10 μM-4,4'-di-isothiocyanostilbene-2,2'-disulphonic acid (DIDS).

Some traces from such experiments are shown in Fig. 2. At 15°C erythrocytes exposed to 15 mM-pyruvate exhibited a Cl$^-$ efflux that could be abolished by α-cyano-4-hydroxycinnamate. The rate of Cl$^-$ efflux observed was 3.71±0.09 ng-ions/min per μl of intracellular space, which is similar to the rate of pyruvate uptake measured under these conditions, 4.74±0.06 nmol/min per μl of intracellular space (means ±S.E.M. of four observations).

In Fig. 2 and Table 3 the efflux of Cl$^-$ associated with the uptake of a variety of anions is shown, all of which are substantially inhibited by α-cyano-4-hydroxycinnamate (2 mM). Pyruvate, L-lactate, DL-β-hydroxybutyrate and acetoacetate may all enter the

Table 3. *Cl⁻ efflux from erythrocytes induced by various carboxylate anions*

Initial rates of Cl^- efflux were measured after addition of the carboxylate anion at 15 mM as described in Fig. 2. The temperature was 15°C and the haematocrit about 50%. The intracellular volume was determined separately by using ^3H_2O and [U-^{14}C]sucrose as described under 'Methods'. Values are the mean of two observations agreeing within 10%.

	Cl⁻ efflux (ng-ions/min per μl of intracellular space)	
Carboxylate anion added	Control	With 2 mM-α-cyano-4-hydroxycinnamate
Pyruvate	3.75	<0.3
Lactate	1.80	<0.3
Acetate	2.7	7.5
Formate	>50	12
Bicarbonate	>50	22
Oxamate	7.5	0.4
Monochloroacetate	12.0	0.4
Dichloroacetate	6.0	<0.3
Trichloroacetate	<0.3	<0.3
3-Chloropropionate	11.0	6.0
2-Chloropropionate	2.25	<0.3
2,2′-Dichloropropionate	0.3	<0.3
Difluoroacetate	4.5	<0.3
DL-β-Hydroxybutyrate	0.50	<0.3
Acetoacetate	0.68	<0.3
Glyoxylate	2.39	<0.3
Glycollate	3.84	<0.3
Phenylpyruvate	0.53	<0.3
2-Oxo-4-methylpentanoate	1.95	<0.3

erythrocyte in exchange for Cl^- ions in this way, their rate of entry being relatively slow compared with that of some of the halogenated carboxylates such as monochloroacetate, dichloroacetate and difluoroacetate. Acetate caused a much faster rate of Cl^- efflux, as shown in Fig. 2(*a*), but this could be inhibited substantially by the inhibitor of carbonic anhydrase, acetazolamide (0.1 mM). This inhibitor was without effect on the Cl^- efflux caused by lactate, pyruvate and all the other aliphatic carboxylates mentioned in Table 3 with the exception of 3-chloropropionate. The inhibition of acetate-induced Cl^- efflux by acetazolamide supports the work of others (Scarpa *et al.*, 1970; Deuticke, 1972; Aubert & Motais, 1975), who have shown that acetate and other weak acids enter the erythrocyte as the free acid. Protons may then be lost by diffusion of CO_2 from the cell followed by its re-entry as HCO_3^- in exchange for Cl^- ions. This process requires the operation of carbonic anhydrase which is inhibited by acetazolamide. α-Cyano-4-hydroxycinnamate (2 mM) and 4,4′-di-isothiocyanostilbene-2,2′-disulphonic acid inhibit the process by inhibiting HCO_3^-/Cl^- exchange (Figs. 2*a* and 2*b*). The proton uptake associated with acetate uptake could be observed by measuring the rise in the extracellular pH as described under 'Methods'. As reported by others (Deuticke, 1972; Aubert & Motais, 1975), it was enhanced by blocking the Cl^- carrier. However no proton uptake was observed when pyruvate and lactate were taken up by erythrocytes, even in the presence of inhibitors of Cl^- transport. Thus pyruvate and lactate entry into the erythrocyte does not involve transport of H^+ or OH^- ions across the membrane, in contrast with the mitochondrial pyruvate transport mechanism (Halestrap, 1975), but rather the countertransport of Cl^- ions.

Isothiocyano derivatives of stilbene have been shown to be extremely potent inhibitors of Cl^- exchange across the erythrocyte cell membrane and to bind to a specific transmembrane protein (Cabantchik & Rothstein, 1972, 1974*a,b*). Fig. 2 shows that inhibition of Cl^- efflux from the erythrocyte induced by a variety of anions added extracellularly can be totally inhibited by very low concentrations of 4,4′-di-isothiocyanostilbene-2,2′-disulphonic acid (<10 μM). Pyruvate- and lactate-induced Cl^- efflux is also inhibited by this compound, and in Fig. 3 I show that the sensitivity of inhibition is the same for pyruvate as for a variety of other anions. Adding increasing concentrations of the inhibitor appears to titrate out the Cl^- efflux linearly, the concentration of inhibitor required to give 95% inhibition being directly proportional to the number of erythrocytes present (Fig. 3*a*). Another stilbene derivative, 4-acetamido-4′-isothiocyanostilbene-2,2′-disulphonic acid, also known to inhibit the erythrocyte Cl^- carrier (Cabantchik & Rothstein, 1972) titrated out Cl^- efflux along the same line as 4,4′-di-isothiocyano-
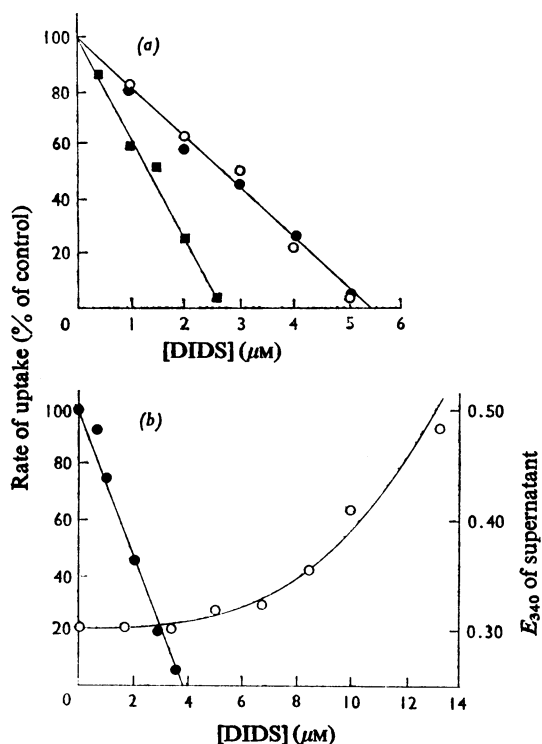
Fig. 3. *Inhibition of Cl⁻ efflux by isothiocyanostilbene derivatives*

Cl⁻ efflux from erythrocytes was measured after addition of the required anion as described for Fig. 2. The number of erythrocytes/ml was 3.3×10^9 ($\bullet$, $\circ$) and 1.15×10^9 ($\blacksquare$) in (a), and 1.7×10^9 in (b). Efflux of Cl⁻ was induced by pyruvate ($\circ$) or bicarbonate ($\bullet$) in (a), and F⁻ in (b). In (b) the E_{340} of the supernatant ($\circ$) was measured after centrifugation (12000g-min). The amount of 4,4'-di-isothiocyanostilbene-2,2'-disulphonic acid (DIDS) required to inhibit Cl⁻ efflux totally was 1.04×10^6 molecules/cell in (a), and 1.29×10^6 molecules/cell in (b).
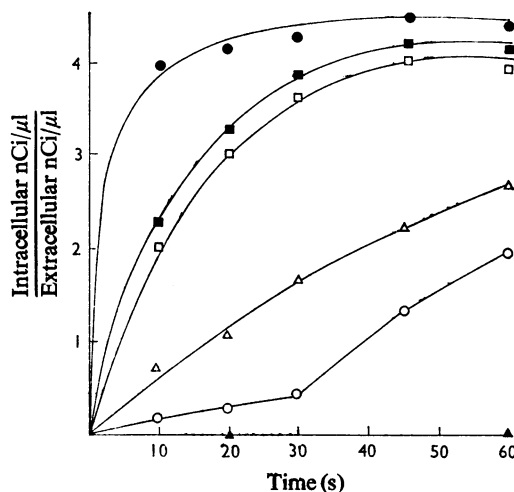

Fig. 4. *Uptake of $^{36}Cl^-$ into erythrocytes*

The experimental conditions were similar to those of Table 1, except that the temperature was 2.5°C and the uptake started by the addition of 2mM-Na^{36}Cl (0.08μCi/ml) and terminated by addition of 40μM inhibitor (4,4'-di-isothiocyanostilbene-2,2'-disulphonic acid) at the required time. Other additions were none ($\bullet$), inhibitor, 3μM ($\blacksquare$), inhibitor (3μM) and 250μM-α-cyano-4-hydroxycinnamate ($\triangle$), inhibitor (3μM) and 250μM-picrate ($\circ$), inhibitor (3μM) and 20μM-pyruvate ($\square$), and inhibitor, 40μM ($\blacktriangle$). Uptake is expressed as the ratio (intracellular radioactivity/μl)/(extracellular radioactivity/μl).

stilbene-2,2'-disulphonic acid. Extrapolation of the results of six such experiments using a variety of anions to induce Cl⁻ efflux gave a value of $1.19 \times 10^6 \pm 0.09 \times 10^6$ molecules of inhibitor bound per cell (mean±S.E.M. of six observations) to give 100% inhibition. Evidence that all the inhibitor was bound is given in Fig. 3(b), where E_{340} of the supernatant was measured after centrifugation to remove the erythrocytes. Only after 100% inhibition was reached did the E_{340} rise, indicating the presence of the inhibitor free in solution. The quantity of inhibitor required to inhibit transport totally is very similar to the number of molecules of the transmembrane protein involved in Cl⁻ transport (about 1×10^6/cell) and which bind isothiocyanostilbene derivatives (Cabant-

chik & Rothstein, 1972; 1974a,b). Direct measurement of the covalent binding of 4,4'-di-isothiocyanostilbene-2,2'-disulphonic acid to the erythrocyte membrane gave a value of only 3×10^5 inhibitor molecules bound/cell (Cabantchik & Rothstein, 1974a; Ho & Guidotti, 1975). However, Cabantchik & Rothstein (1972) reported that some inhibition of Cl⁻ efflux can be produced by isothiocyanostilbene derivatives without covalent binding, since part of the inhibition can be reversed by addition of albumin. I was able to observe similar relief of inhibition of Cl⁻ efflux from erythrocytes by albumin (10mg/ml). Inhibition in the presence of 10μM inhibitor was decreased from 100 to 78% by addition of albumin when 4,4'-di-isothiocyanostilbene-2,2'-disulphonic acid was the inhibitor, and to 60% in the case of 4-acetamido-4'-isothiocyanostilbene-2,2'-disulphonic acid.

The similarity of the inhibition by isothiocyanostilbene derivatives of the Cl⁻ efflux induced by pyruvate, lactate and bicarbonate to their inhibition of Cl⁻/Cl⁻ exchange reported by others (Cabantchik & Rothstein, 1972, 1974a,b), suggests that pyruvate and
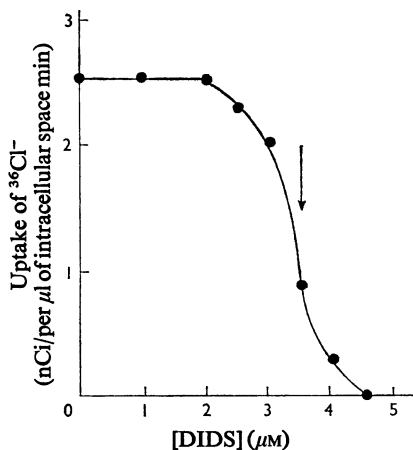
Fig. 5. *Inhibition of Cl^-/Cl^- exchange by 4,4'-di-isothio-cyanostilbene-2,2'-disulphonic acid*

Conditions of uptake were identical with those of Fig. 4, uptake being terminated at 30s. 4,4'-Di-isothiocyano-stilbene-2,2'-disulphonic acid (DIDS) was added at the concentration shown. The concentration of cells was 1.8×10^9/ml. The arrow represents at least 90% inhibition of Cl^- efflux measured on the chloride electrode.

lactate may utilize the Cl^- carrier to enter the erythro-cyte. To confirm the results obtained by using the Cl^- electrode, experiments were performed with $^{36}Cl^-$. By using the Cl^- electrode it was shown that 4,4'-di-isothiocyanostilbene-2,2'-disulphonic acid totally and immediately inhibited Cl^- efflux; it was therefore used as the quenching agent for 'inhibitor-stop' experiments on $^{36}Cl^-$ uptake into erythrocytes. This exchange process had an extremely rapid time-course, appeared to be almost complete within 10s at 2.5°C (Fig. 4), and was abolished by $10 \mu M$-4,4'-di-isothiocyanostilbene-2,2'-disulphonic acid. Addition of enough of this inhibitor to produce about 75% inhibition of HCO_3^-/Cl^- exchange allowed the time-course of $^{36}Cl^-$ uptake to be studied. As found by using the Cl^- electrode, picrate, a well-known inhibi-tor of chloride transport (Gunn & Tosteson, 1971; Wieth, 1970) and α-cyano-4-hydroxycinnamate showed substantial inhibition, but pyruvate did not. Fig. 5 shows that the sensitivity of Cl^-/Cl^- exchange to inhibition by 4,4'-di-isothiocyanostilbene-2,2'-di-sulphonic acid is similar to that shown for the in-hibition of Cl^- efflux observed in experiments using the Cl^- electrode. However, an exact titration was not possible, since initial rates of exchange were too fast to measure until inhibition was substantial. These results confirm the reliability of the Cl^- electrode as a means for obtaining an accurate and continuous record of Cl^- transport.

Effects of inhibitors of Cl^- transport on the transport of pyruvate and lactate

At concentrations of 4,4'-di-isothiocyanostilbene-2,2'-disulphonic acid that totally inhibited Cl^- efflux from erythrocytes, pyruvate and lactate uptake into the cells still occurred, but at an inhibited rate. The kinetics of the inhibition of the transport of pyruvate and lactate into erythrocytes under these conditions are shown in Fig. 6. For both substrates inhibition appeared to be uncompetitive, but with departures from linearity in the Lineweaver–Burk plot at high substrate concentrations. Similar, but less pronounced, uncompetitive patterns of inhibition were also observed in the presence of other inhibitors or substrates of the Cl^- carrier such as picrate, bi-carbonate, chloride, formate and fluoride (see Table 1). Such a pattern of inhibition would be expected if two transport mechanisms for pyruvate and lactate exist, one with a high K_m and high $V_{max.}$, inhibited by inhibitors of Cl^- transport, and the other with a low K_m and low $V_{max.}$, independent of Cl^- transport (see Neame & Richards, 1972). A theoretical plot showing the kinetics expected under these circum-stances is shown in Fig. 6(c), which also shows the effect of inhibiting the high-$V_{max.}$ high-K_m carrier. This theoretical plot shows striking similarities to the kinetics actually observed for the uptake of lactate and pyruvate into the erythrocyte, and implies that both the Cl^- carrier and a Cl^--independent carrier are utilized. The Cl^--independent carrier would have a low K_m (about 1.5mM for pyruvate and 10mM for lactate) and low $V_{max.}$ (about 2nmol/min per μl of intracellular space for both substrates). The Cl^- carrier, however, would have a high K_m (about 300mM) and a high $V_{max.}$ (about 75nmol/min per μl of intra-cellular space) for both substrates. Evidence that this is so is described below.

Nature of the Cl^--independent transport of pyruvate and lactate

Since no protons were involved in pyruvate and lactate uptake into the erythrocyte, it would seem likely that the Cl^--independent transport mechanism would involve exchange with another anion. The blood used in these experiments was normally kept in the presence of glucose, which suggested that the cells might contain a large amount of lactate formed by glycolysis. This was found to be true, and lactate concentrations as high as 20mM could be measured inside the cell. Pyruvate might therefore exchange for lactate in 4,4'-di-isothiocyanostilbene-2,2'-di-sulphonic acid-treated cells. The experiment reported in Fig. 7 would indicate that this is possible. Cells were washed three times with 5vol. of 0.15M-NaCl, incubation for 1h at 37°C being included in each wash. They were then washed and suspended in citrate medium as usual and treated with $10 \mu M$-4,4'-di-
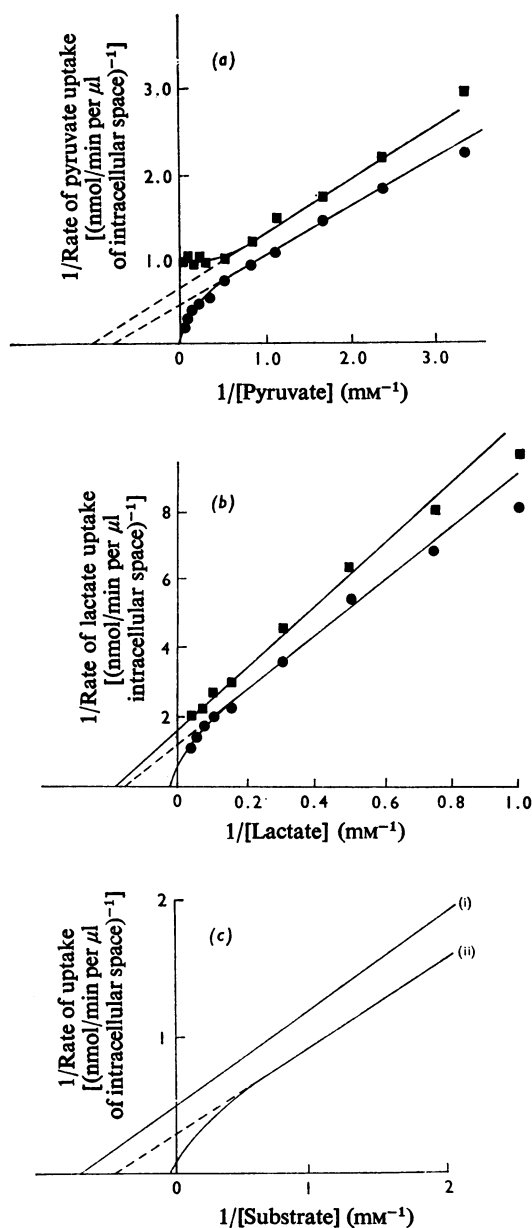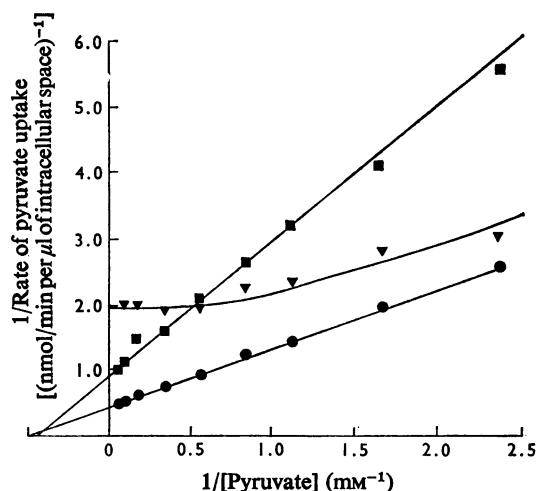
Fig. 7. *Effect of intracellular lactate on the uptake of pyruvate into 4,4'-di-isothiocyanostilbene-2,2'-disulphonic acid-treated cells*

Erythrocytes were either washed three times with 0.15 M-NaCl, each wash including an incubation for 1 h at 37°C, or incubated once in 150 mM-sodium DL-lactate for 1 h at 37°C before washing twice in citrate buffer. Cells were then suspended in citrate buffer (25% haematocrit) and the kinetics of pyruvate uptake studied as described in Table 1. Saline-treated cells (▼), lactate-treated cells (●), lactate-treated cells+300 μM-picrate (■).

Fig. 6. *Effect of 4,4'-di-isothiocyanostilbene-2,2'-disulphonic acid on uptake of pyruvate and lactate into erythrocytes*

The conditions used for expts. (*a*) and (*b*) were identical with those used in Table 1. For both experiments either no addition was made (●) or 10 μM-4,4'-di-isothiocyanostilbene-2,2'-disulphonic acid (■) was added. In (*c*) a theoretical plot is given that shows the predicted kinetics for (i) a carrier with $K_m = 1.5$ mM and $V_{max.} = 2$ nmol/min per μl of intracellular space, and (ii) the same carrier in the presence of a second carrier, with $K_m = 300$ mM and $V_{max.} = 75$ nmol/min per μl.

isothiocyanostilbene-2,2'-disulphonic acid. Another group of cells were incubated in 0.15 M-sodium DL-lactate before being washed and suspended in citrate medium containing 10 μM-4,4'-di-isothiocyanostilbene-2,2'-disulphonic acid. The kinetics of the uptake of pyruvate were measured in each case (Fig. 7). In saline-washed cells, very little increase in the rate of pyruvate uptake was observed with increasing pyruvate concentrations, whereas the lactate-treated cells showed normal kinetics without the plateau at high pyruvate concentrations that had been observed earlier (Fig. 6*a*). The K_m was 2.13 ± 11 mM and the $V_{max.}$ 2.32 ± 0.04 nmol/min per μl of intracellular space, picrate showing non-competitive inhibition with a K_i of 0.265 ± 0.019 mM. These kinetic parameters are very similar to those obtained at low pyruvate concentrations (<4 mM) in the absence of 4,4'-di-isothiocyanostilbene-2,2'-disulphonic acid reported in Table 2. This is probably because lactate was not washed out of these cells before pyruvate uptake was studied, and only relatively low substrate concentrations were used. The plateau in the rate of pyruvate uptake seen at high pyruvate concentrations when Cl⁻ transport is inhibited by 4,4'-di-isothiocyanostilbene-2,2'-disulphonic acid appears to be caused by the transport of lactate out of the cell

Table 4. *Lactate efflux from preloaded erythrocytes*

Erythrocytes were preloaded with L-lactate by storing in the presence of glucose for 4 weeks at 4°C, and then washed with 5 vol. of citrate buffer containing 4,4'-di-isothiocyanostilbene-2,2'-disulphonic acid (10 μM) at 0°C. The cells were separated by centrifugation and 2 ml of cells was added to 4 ml of citrate buffer at 10°C containing 4,4'-di-isothiocyanostilbene-2,2'-disulphonic acid (10 μM) and the relevant additional anion at 10 mM. After 3 min, 1 ml samples were centrifuged at 15000g for 1 min, and 0.5 ml of supernatant, acidified with 0.1 ml of HClO₄ (20%, v/v) and centrifuged. The resulting supernatant (15000g-min) was assayed for lactate by the method of Bücher *et al.* (1963). Control experiments in which the erythrocytes were added to citrate buffer at 0°C containing only 4,4'-di-isothiocyanostilbene-2,2'-disulphonic acid (10 μM) and centrifuged immediately were performed to correct for the carry-over of extracellular lactate. The total intracellular lactate was measured by an identical procedure, except that citrate buffer was replaced by water, in which the cells lysed. The intracellular volume was measured as described under 'Methods'. Results are means±S.E.M. of four observations. Statistical significance was assessed by Student's '*t*' test. *$P < 0.01$ when compared with control incubation; **$P < 0.01$ when compared with pyruvate incubation.

Additions	Lactate output in 3 min (nmol/μl of intracellular space)
None	0.154 ± 0.021
Pyruvate	1.187 ± 0.035*
Pyruvate + 1.0 mM-α-cyano-cinnamate	0.180 ± 0.015**
Bicarbonate	0.245 ± 0.028
Monochloroacetate	1.520 ± 0.057*
Dichloroacetate	1.500 ± 0.015*
DL-β-Hydroxybutyrate	0.522 ± 0.005*
Acetoacetate	0.557 ± 0.047*
Phenylpyruvate	0.200 ± 0.026
2-Oxo-4-methylpentanoate	0.446 ± 0.058*
Acetate	0.986 ± 0.005*
Total intracellular lactate	17.20 ± 0.194

becoming slower than the potential rate of pyruvate entry.

In order to assess what anions other than lactate can exchange with pyruvate on the 4,4'-di-isothiocyanostilbene-2,2'-disulphonic acid-insensitive carrier, the experiment reported in Table 4 was performed. Erythrocytes containing about 20 mM-lactate by virtue of their metabolism of glucose, were suspended in media containing 4,4'-di-isothiocyanostilbene-2,2'-disulphonic acid (10 μM) and various carboxylate anions. Those carboxylate anions able to use the 4,4'-di-isothiocyanostilbene-2,2'-disulphonic acid-insensitive carrier should cause an efflux of lactate as they enter the cell. As expected, pyruvate caused a substantial efflux of lactate which could be blocked by α-cyanocinnamate. Monochloroacetate,

dichloroacetate and acetate were good substrates, whereas 2-oxo-4-methylpentanoate and the ketone bodies acetoacetate and DL-β-hydroxybutyrate were less effective. Phenylpyruvate and bicarbonate did not stimulate lactate efflux at all. The Cl⁻-independent carrier appears to have a fairly broad specificity for carboxylates, although bicarbonate and phenylpyruvate seem to be outside these limits.

Nature of pyruvate transport on the Cl⁻ carrier

The K_m of the Cl⁻ carrier for pyruvate and lactate appeared to be very high (>250 mM) and proved impractical to measure. It was of interest, however, to establish the nature of the inhibition of pyruvate transport on this carrier by α-cyano-4-hydroxycinnamate. This was attempted by looking at the kinetics of chloride efflux induced by bicarbonate.

This process has been studied extensively, since it is essential for the working of the 'chloride shift' which greatly enhances the ability of the blood to transport CO_2 and HCO_3^- (see Deuticke, 1972; Dalmark, 1972; Wieth, 1972). However, it is extremely rapid even at 0°C and previous kinetic data have been limited by the lack of a continuous record of Cl⁻-flux measured. The use of the Cl⁻ electrode overcomes this problem and allows better kinetic experiments to be performed. Varying the concentration of added bicarbonate gave no change in the rate of Cl⁻ efflux, so it was assumed that transport of Cl⁻ rather than of bicarbonate was rate-limiting. By preincubating erythrocytes in iso-osmotic solutions of sodium chloride and sodium acetate, before preparation of the cells in Cl⁻-free medium, it was possible to alter the intracellular Cl⁻ concentration, which could be calculated from the Donnan equilibrium ($Cl^-_{in}/Cl^-_{out} = 0.7$; Garrahan, 1972). The rate of Cl⁻ efflux induced by bicarbonate (20 mM) at various intracellular Cl⁻ concentrations followed Michaelis–Menten kinetics. At 2.5°C the K_m for Cl⁻ was 23.8 ± 2.1 mM and the $V_{max.}$ 204 ± 6 ng-ions/min per μl of intracellular space. The values for the K_m and $V_{max.}$ obtained in this experiment agree very well with those obtained by using Cl⁻/Cl⁻ exchange in iso-osmotic acetate at 0°C (R. B. Gunn, J. O. Wieth & O. C. Tosteson, quoted in Wieth, 1972; Dalmark, 1975). However, the effects of inhibitors on this system could not be studied satisfactorily because the inhibitors had to be added on the outside of the cell, whereas the substrate (chloride) was present on the inside. To circumvent this problem the kinetics of the efflux of Cl⁻ induced by F⁻ were studied, since under these conditions the transport of F⁻ and not Cl⁻ appeared to be rate-limiting. This allowed both the rate-limiting substrate, F⁻, and the inhibitor, to be added on the same side of the membrane. The results of these experiments are shown in Fig. 8. The K_m for F⁻ was 99 ± 6 mM and the $V_{max.}$ 75 ± 3 nmol/min per μl of intracellular space. α-Cyano-4-hydroxy-
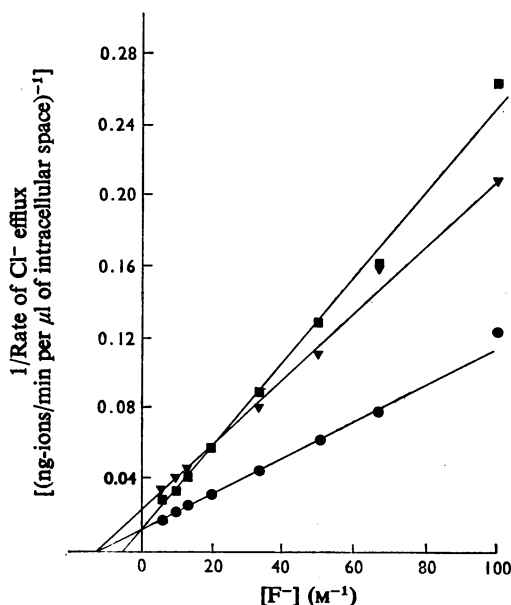
Fig. 8. *Kinetics of fluoride entry into erythrocytes*

Erythrocytes were prepared as described for Table 1. Cl^- efflux was measured at 2.5°C after addition of NaF at the concentration required, and rates of efflux were determined as described for Fig. 2; corrections were made for the volume of NaF added. Other additions were as follows: none (●), 120μM-α-cyano-4-hydroxycinnamate (▼), 90μM-picrate (■). K_m, $V_{max.}$ and K_i values are given in the text.

cinnamate appeared to inhibit the process non-competitively with a K_i of $125\pm18\mu$M, whereas picrate inhibited competitively with a K_i of $70\pm4\mu$M. Picrate and similar aromatic anions have been known as inhibitors of anion transport into erythrocytes for some time (Gunn & Tosteson, 1971; Wieth, 1970) and are thought to bind tightly to the positively charged groups involved in anion transport. Pyruvate and lactate at concentrations as high as 50mM showed no consistent inhibition of F^--induced Cl^- efflux, except at very low (<20mM) concentrations of F^-. Neither was inhibition of Cl^-/Cl^- exchange observed in the presence of pyruvate (Fig. 4). These results agree well with the predicted high K_m of the Cl^- carrier for these substrates (see Fig. 6). Further evidence supporting the hypothesis that both a high-K_m Cl^--dependent and a low-K_m Cl^--independent carrier are present comes from comparing the rate of pyruvate uptake with that of the accompanying Cl^- efflux under the same conditions. Pyruvate uptake proceeded quite fast at 15°C (1.36±0.05nmol/min per μl) (mean±S.E.M. of four observations), but Cl^- efflux was too slow to be measured (<0.3ng-ions/min

per μl). However, at 15mM-pyruvate the two processes occurred at similar rates (4.47±0.06nmol/min per μl and 3.71±0.09ng-ions/min per μl respectively).

Relationship between the Cl^--dependent and -independent carriers

It is noteworthy that both the Cl^--dependent and the Cl^--independent carriers show some similarities. Both are inhibited by non-specific amino reagents such as fluorodinitrobenzene, but not by thiol reagents (Table 2 and Sachs et al., 1975). Erythrocytes exposed to the specific amino reagent 4,4'-di-isothiocyanostilbene-2,2'-disulphonic acid, which inactivates the transport of pyruvate and lactate on the Cl^- carrier, could be made totally impermeable to these substrates by incubation with 1mM-fluorodinitrobenzene under the conditions given in Table 2.

Both carriers are also inhibited by α-cyano-4-hydroxycinnamate and picrate. The nature of the inhibition is different, however, α-cyano-4-hydroxycinnamate showing competitive inhibition of the Cl^--independent carrier, but non-competitive inhibition of the Cl^--dependent carrier; picrate shows the reverse pattern of inhibition. It therefore seems possible that both carrier functions are contained in the same membrane protein but at different sites, inhibition of one site also having an inhibitory effect on the other site. It is known that mild Pronase treatment of erythrocytes cleaves the 95000-mol.wt. protein involved in Cl^- transport to yield a 65000-mol.wt. fragment that is still active in anion transport and inhibited by 4,4'-di-isothiocyanostilbene-2,2'-disulphonic acid (Passow, 1971; Cabantchik & Rothstein, 1974b). Similar experiments showed that Pronase treatment also had little or no effect on Cl^--independent pyruvate uptake. That Pronase had cleaved the 95000-mol.wt. protein was confirmed by running sodium dodecyl sulphate/polyacrylamide gels of 'ghost' membranes from control and Pronase-treated cells. Thus both carriers appear to be insensitive to mild Pronase treatment. It is possible that various amino groups are available on the 95000-mol.wt. protein and that some of these are involved in general anion transport and others in carboxylate transport. Fluorodinitrobenzene must inactivate both sets of sites, whereas 4,4'-di-isothiocyanostilbene-2,2'-disulphonic acid inactivates only those involved with Cl^- transport. Proof as to whether both sites are on the same protein must await reconstitution of the pure 95000-mol.wt. protein into artificial membranes and establishing whether both carrier activities can be restored. Rothstein et al. (1975) have gone some way towards reconstituting such a system.

General conclusions

The present results suggest the existence of two anion carriers in the erythrocyte. One of these has a

Table 5. *Uptake of picrate and α-cyano-4-hydroxycinnamate by erythrocytes*

Erythrocytes were incubated in citrate buffer (haematocrit about 20%) at 20°C with picrate (0.4mM) for 2.5min or with α-cyano-4-hydroxycinnamate (0.4mM) for 10min, before centrifugation. The supernatant was diluted tenfold with water before measurement of the E_{355} (picrate) or E_{340} (α-cyano-4-hydroxycinnamate). The uptake of both compounds was calculated from the fall in extinction of the supernatant when compared with control incubations in the absence of cells, and from the intracellular and extracellular volumes measured as described under 'Methods'. The molar extinction coefficient of picrate at 355nm was 14×10^3 and that of α-cyano-4-hydroxycinnamate at 340nm was 20.9×10^3 litre·mol^{-1}·cm^{-1}. Values are given as the mean±s.E.M. of four observations. Statistical significance was measured by Student's 't' test. *$P < 0.01$ when compared with incubations in the absence of 4,4'-di-isothiocyanostilbene-2,2'-disulphonic acid.

| Substrate	Final external concn. (mM)	Uptake (nmol/μl of intracellular space)	Concentration ratio (inside/outside)
Picrate	0.072 ± 0.005	3.64 ± 0.05	51.2 ± 4.2
Picrate+4,4'-di-isothiocyanostilbene-2,2'-disulphonic acid (10μM)	0.335 ± 0.002*	1.19 ± 0.02*	3.56 ± 0.07*
α-Cyano-4-hydroxycinnamate	0.344 ± 0.002	1.08 ± 0.02	3.08 ± 0.07
α-Cyano-4-hydroxycinnamate+4,4'-di-isothiocyanostilbene-2,2'-di-sulphonic acid (10μM)	0.431 ± 0.001*	0.31 ± 0.03*	0.71 ± 0.07*

particularly high affinity for Cl$^-$ and HCO$_3^-$ ions but may also transport carboxylate anions such as pyruvate, lactate and halogenated acids, albeit with a very high K_m, probably of the order of several hundred mM. This carrier is inhibited competitively by picrate and non-competitively by α-cyano-4-hydroxycinnamate; both inhibitors bind extremely tightly to the transporter, although they may be transported slowly (Table 5). Isothiocyano stilbene derivatives such as 4,4'-di-isothiocyanostilbene-2,2'-disulphonic acid and 4-acetamido-4'-isothiocyanostilbene-2,2'-disulphonic acid appear to be able to inhibit this carrier totally, requiring about 1 molecule of inhibitor per molecule of the 95000-mol.wt. protein present in the membrane believed to be associated with transport of chloride, sulphate and phosphate (Cabantchik & Rothstein, 1972, 1974a,b; Ho & Guidotti, 1975).

Some pyruvate and lactate may enter the cell when this Cl$^-$-independent carrier is totally inhibited. The K_m values for lactate and pyruvate on this carrier were about 9 and 2mM respectively, and the K_i values of lactate against pyruvate and vice versa were very much the same as their K_m values, implying that the K_m represented the dissociation constant of the carrier–substrate complex. α-Cyano-4-hydroxycinnamate and its analogues are powerful competitive inhibitors of this transporter (K_i about 50μM), whereas picrate appears to be a non-competitive inhibitor. A large variety of small carboxylate anions are able to use the carrier, but bicarbonate is not a substrate.

The affinity of the low-K_m carrier for pyruvate and lactate appeared to be about two orders of magnitude greater than that of the Cl$^-$ carrier, although the $V_{max.}$ wat very much lower. Thus at low lactate and pyruvate concentrations this carrier would be used in preference to the other. However, both carriers only catalyse

exchange transport of one anion for another. Hence the low-K_m carrier can only catalyse such exchanges as pyruvate for lactate or acetoacetate for β-hydroxybutyrate. These exchanges may be of use in transferring reducing power from the cytoplasm to the plasma or vice versa, but they are of no use in achieving a net loss of lactic acid from the cell. This process is of course of vital importance in the erythrocyte, which produces lactate in abundance during anaerobic metabolism of glucose. Muscle cells may be faced with the same problem during violent exercise, when anaerobic oxidation of glucose is required. Similarly, but in reverse, net uptake of lactic acid by the liver is required during the operation of the Cori cycle for re-synthesis of glucose from lactate. I have been unable to show any proton uptake associated with transport of lactate or pyruvate, which agrees with the work of others (Deuticke, 1972; Aubert & Motais, 1975). It seems therefore that in the erythrocyte lactate must normally leave the cell on the Cl$^-$ carrier in exchange for HCO$_3^-$, which is then lost as CO$_2$, effectively taking a proton with it as shown in Scheme 1.

Whether such a mechanism also occurs in liver and muscle, and whether the 95000-mol.wt. protein that is responsible for this process in the erythrocyte exists in the cell membranes of these other tissues, must await further investigation. Work by Lamers (1975) and Lamers & Hülsmann (1975) on pyruvate transport into isolated intestinal cells, indicates the existence of an anionic counter-transport system for pyruvate very similar to the low-K_m carrier described here rather than the high-K_m carrier. However, it is noteworthy that in the working gastrocnemius muscle of the dog the rate of lactate permeation across the membrane is increased when plasma HCO$_3^-$ ion concentrations are increased (Hirche *et al.*, 1975). Cer-
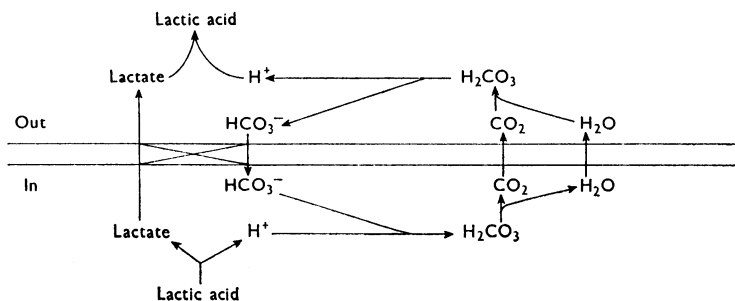
Scheme 1. Net transport of lactic acid out of the erythrocyte

tainly muscle cells are permeable to the HCO_3^- ion and this seems to be involved in regulating the pH of the cell (Adler et al., 1965; Stegemann, 1964). Preliminary experiments with isolated rat liver cells have shown inhibition of gluconeogenesis from lactate by 50 μM-4,4'-di-isothiocyanostilbene-2,2'-disulphonic acid but these experiments have been hampered by the presence of albumin (20mg/ml) in the medium, which binds the inhibitor very tightly (A. P. Halestrap & J. Mendes Mourão, unpublished work). In Ehrlich ascites cells there is some evidence that transport of SO_4^{2-} and Cl^- ions occurs on the same carrier (Levinson & Villereal, 1975), but only sulphate transport has been shown to be inhibited by amino reagents (Levinson & Villereal, 1973). Spencer (1975) reported that lactate and other α-substituted carboxylates enter the Ehrlich ascites cells with a proton, and that entry may be inhibited by high concentrations of α-cyanocinnamate.

If other tissues do have a Cl^-/HCO_3^- carrier similar to that of the erythrocyte, several interesting consequences emerge. The intracellular concentration of Cl^- in heart and muscle is extremely low, of the order of 5–25 mM (Waddell & Butler, 1959; Carmeliet, 1961; Zachariah, 1961; Adler et al., 1965), when compared with the plasma Cl^- concentration (145mM). If Cl^-/HCO_3^- exchange is possible, it would be predicted that a HCO_3^- gradient similar to that of Cl^- would be established across the cell membrane. This in turn would produce a pH gradient, since it was shown that the pH of the muscle cell is dependent on the HCO_3^- concentration (Adler et al., 1965). The net result of this argument is that the existence of a Cl^- gradient across a cell containing a mechanism for rapid Cl^-/HCO_3^- exchange necessitates the existence of a pH gradient. In erythrocytes, I have demonstrated this (A. P. Halestrap, unpublished work). In heart and muscle, therefore, with their low intracellular Cl^- concentrations, a low intracellular pH would be expected. Direct measurement of the pH with a micro-pH-electrode gave pH values of about 6.0 (Carter et al., 1967a,b), as have observations with

the cationic pH-sensitive dye 9-aminoacridine (D. W. Deamer, unpublished work). Measurements with the anionic pH indicator 5,5-dimethyl-2,4-oxazolidinedione have, however, given intracellular pH values nearer 7.0 (Waddell & Butler, 1959; Adler et al., 1965; Clancy & Brown, 1966).

Watts (1969) showed that in the perfused heart exposed to chloroacetate, lactate, pyruvate or acetoacetate, little or no internal substrate could be detected under normal conditions. However, if Cl^-- and HCO_3^--free perfusion medium (containing sulphate and imidazole) was used, significant internal concentrations of the substrates accumulated. Such results would be expected if these acids (HX, where X^- is the anion) entered the cell in exchange for HCO_3^- on a general anion transporter as I have suggested, since at equilibrium:

$$\frac{[X^-]_{in}}{[X^-]_{out}} = \frac{[HCO_3^-]_{in}}{[HCO_3^-]_{out}} = \frac{[Cl^-]_{in}}{[Cl^-]_{out}}$$

Thus the concentration ratio of internal to external carboxylate anion will be low when normal perfusion medium is used, since $[Cl^-]_{in}/[Cl^-]_{out}$ is very low. However, when Cl^--free medium is used $[Cl^-]_{in}/[Cl^-]_{out}$ will be very high and thus internal accumulation of carboxylate anions is to be expected. Watts (1969) was also able to demonstrate that in perfused hearts the equilibrium position of $[lactate]_{in}/[lactate]_{out}$ must be considerably less than unity. Hearts perfused with 9mM-lactate and 10mM-pyruvate showed net loss of lactate to the perfusion medium, even though the internal lactate concentration was only about 30% of the external concentration.

There is therefore good reason to suppose that a general anion transporter, such as is found in the erythrocyte, may be present in heart and muscle. This would have far-reaching consequences in the regulation of cell pH and intracellular metabolite concentrations. It would also explain why pharmacological agents such as dichloroacetate and α-cyano-4-hydroxycinnamate are much less potent on whole

cell preparations than with isolated mitochondria or purified enzymes (Whitehouse *et al.*, 1974; Halestrap & Denton, 1975). Direct evidence for the presence of a general anion transporter in other tissues has yet to be found.

The nature of the low-K_m carrier for pyruvate requires further elucidation. Like the chloride carrier it is inhibited by amino reagents (but not 4,4'-di-isothiocyanostilbene-2,2'-disulphonic acid) and not by thiol reagents or Pronase treatment. This latter observation is of particular interest, since it implies that the active part of the protein is not accessible to large molecules such as Pronase. The 95000-mol.wt. protein is attacked by Pronase to yield a 65000-mol.wt. protein which is still active with respect to transport of Cl^- and SO_4^{2-} (Boxer *et al.*, 1974; Cabantchik & Rothstein, 1974b). The topography of this protein in the membrane and the effects of Pronase and other proteolytic enzymes on it were studied extensively by Jenkins & Tanner (1975). These workers conclude that the molecule is 'S'-shaped, crossing the membrane twice, only the external portion being removed by Pronase treatment. Such a molecule would be well suited for transporting ions across the membrane, and it is possible that the same protein contains two sites, one for anions in general and Cl^- and HCO_3^- ions in particular, the other for carboxylates such as pyruvate and lactate. If the sites were fairly close it might explain why large organic ions such as picrate and α-cyano-4-hydroxy-cinnamate can inhibit both activities, but one competitively and the other non-competitively. The 95000-mol.wt. protein has been isolated from the membrane (Tanner & Boxer, 1972; Steck & Dawson, 1974) and some structural work carried out (Jenkins & Tanner, 1975). Attempts have also been made to insert protein into artificial membranes and increase their sulphate permeability (Rothstein *et al.*, 1975). A combination of such techniques should allow conclusive demonstrations of whether or not both carrier functions are carried on the same polypeptide chain.

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